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Teratogenic effects of amniotic sac puncture: a mouse model

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ABSTRACT

The possibility of an association between chorionic villus sampling (*cvs*) and limb abnormalities has prompted a review of the relevant experimental data. Although a vascular aetiology is favoured by many at present, the possibility exists that a proportion of cases may be caused by oligohydramnios secondary to inadvertent amniotic sac puncture. A mouse model of amniotic puncture syndrome has been developed to study the craniofacial and limb abnormalities produced by this procedure. Pregnant mice were anaesthetised and a laparotomy performed. One uterine horn was exteriorised, and the amniotic sacs punctured through the wall of the uterus with either a 21 gauge or a 25 gauge needle. The conceptuses in the contralateral uterine horn acted as controls. The mice were all killed on d 19 of pregnancy (day of finding a vaginal plug = d 1 of pregnancy) by cervical dislocation, and the morphological features of the embryos examined in detail. In a preliminary study, amniotic sac puncture was carried out on d 12, 13, 14, 15 or 16 of pregnancy, with either a 21 or a 25 gauge needle. Since the highest rates of palatal defects and limb deformities were observed following amniotic sac puncture using a 21 gauge needle, when this procedure was carried out on either d 13 or 14 of pregnancy, the main study was undertaken using a 21 gauge needle on these two days of pregnancy. Of 102 embryos in which amniotic sac puncture was carried out on d 13, 53% survived to d 19. Of the latter, 35% had a cleft palate, 61% had one or more morphologically abnormal limbs, and 43% had an abnormal tail. When amniotic sac puncture was carried out on d 14 of pregnancy, of 83 embryos subjected to this procedure, 81% survived to d 19. Of the latter, 27% had a cleft palate, 39% had one or more morphologically abnormal limbs, and 19% had an abnormal tail. In the controls, of 86 and 61 embryos isolated respectively from the d 13 and 14 mice, the survival rates were 97 and 90%, respectively. Palatal, limb and tail abnormalities were not observed in the control series. Tentative relationships are drawn between *cvs* and amniocentesis-associated abnormalities, the Robin sequence and their rodent phenocopies.

Key words: Cleft palate; limb deformities; tail abnormalities; postural deformities.

INTRODUCTION

A number of recent publications have raised the possibility of an association between first trimester chorionic villus sampling (*cvs*) carried out between days (d) 55 and 65 of gestation and the birth of infants with limb reduction defects, orofacial and other congenital malformations (Planteydt et al. 1986; Christiaens et al. 1989; Kaplan et al. 1990; Editorial, 1991; Firth et al. 1991; Hsieh et al. 1991; Mahoney, 1991; Mastroiacovo & Cavalcanti, 1991; Rodriguez & Palacios, 1991; Burton et al. 1992, 1993; Froster & Baird, 1992; Kuliev et al. 1992; Schloo et al. 1992). The body of opinion (Burton et al. 1992; Brent, 1993; Report of the NICHD Workshop, 1993) is that these abnormalities have a vascular basis, the hypothesis being that removal of a small sample of the chorionic villi may cause (1) fetal blood loss resulting in hypotension, hypoperfusion and anoxia and/or (2), thrombus formation at the site of biopsy with distal embolisation. This view is based on the fact that similar craniofacial defects are induced in rat fetuses following vascular disruption of the pregnant uterus (Brent & Franklin, 1960; Brent, 1990), and limb defects are observed following uterine trauma and clamping of the uterine arteries for 45 min (Webster

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et al. 1987; see also Hoyme et al. 1982). However, it seems unlikely that the analogy between complete cessation of uterine blood flow and *cvs* is a direct one.

Human studies are necessarily very difficult to perform. Quintero et al. (1992) have provided the only direct evidence of a link between placental trauma and haemorrhage in human limbs. Using embryoscopy, they observed haemorrhagic lesions on the scalp, face, thorax and distal limbs of a fetus at 9.5 menstrual wk, following transvaginal *cvs*. Subsequently, they performed embryoscopy before and after *cvs* at 8 to 12 wk, in patients scheduled to have elective terminations of pregnancy, but observed no haemorrhages. In additional studies, they detached the placenta, using a blunt instrument, producing a subchorionic haematoma. Following this radical procedure the fetuses showed petechiae on the head, face and thorax. Any correlation between partial placental detachment and *cvs*, however, is unclear. It is noteworthy that, even in this extreme case of placental trauma, no lesions were observed on the limbs.

The possibility has been raised (Shepard et al. 1991) that inadvertent puncture of the amniotic membranes during *cvs* could account for the limb abnormalities observed. At the end of the 8th wk, amniotic fluid volume is small (Chamberlain et al. 1984; Brace & Wolf, 1989; Gilbert & Brace, 1993): around 10 ml (range 4–40 ml). If the amniotic fluid was lost at this stage, it is unlikely that either patient or clinician would notice. Furthermore, fluid loss might be concealed by slow leakage over a number of hours. Equally, in those mothers who suffer vaginal bleeding after *cvs*, amniotic fluid leakage is likely to remain hidden. In a recent clinical trial, vaginal bleeding was reported in 7% of women after transcervical *cvs* and in 2% after transabdominal *cvs* (MRC Working Party on the Evaluation of Chorionic Villus Sampling, 1991).

Single punctures of the amniotic membranes (with or without active withdrawal of fluid) in the mouse (Trasler et al. 1956; Walker, 1959) and rat (Poswillo & Roy, 1965; Poswillo, 1966, 1968; DeMyer & Baird, 1969; Kino, 1972, 1975; Love & Vickers, 1972; Singh & Singh, 1973; Singh et al. 1974; Kennedy & Persaud, 1977; Houben, 1980, 1984; Houben & Huygen, 1987) have produced both craniofacial and limb abnormalities. These studies have indicated that the critical period for the development of these lesions is comparable to the period of palate closure in man (8–10 wk). This also coincides with the period during which a range of abnormalities associated with *cvs*, termed the oromandibular-limb hypogenesis spectrum, has been observed. It therefore seemed interesting to reevaluate these experimental findings, and develop a murine experimental model, with the particular aim of analysing in detail the incidence and types of disturbances of the palate and limbs induced by this experimental approach.

MATERIALS AND METHODS

The animals used in this study were virgin $(C57BL \times CBA)F_1$ hybrid female mice aged between 8 and 10 wk. The females were caged overnight with $(C57BL \times CBA)F_1$ hybrid males. The presence of a vaginal plug the following morning was taken as evidence that mating had occurred. This defined d 1 of pregnancy, normal delivery in the mouse being on d 20. All mice were kept under controlled environmental conditions (temperature 20°C; relative humidity approximately 50%) and fed a standard pelleted diet (Bantin & Kingman) and water, available ad libitum.

On d 12, 13, 14, 15 or 16 of pregnancy, the mice were given a general anaesthetic (an intraperitoneal injection of 0.016 ml of a 2.5% solution of tribromoethanol (Avertin) per gram of body weight (Hogan et al. 1986)). A midline laparotomy was performed and one of the uterine horns exteriorised, the other remaining undisturbed, acting as a control. The ovary, whether belonging to the left or right side, was identified, and the number and location of embryos and resorption sites recorded. The tip of a 21 or 25 gauge needle (external diameters 0.65 and 0.40 mm, respectively) was passed through the wall of the uterus in the location of each gestational sac, avoiding the location of the placenta and major blood vessels associated with the yolk sac, and allowed to enter the amniotic sac. Care was also taken to avoid contact between the tip of the needle and the embryo. The needle was then withdrawn, allowing an uncontrolled amount of amniotic fluid to leak out. Puncture was considered adequate only when withdrawal of the needle was followed by the escape of a small volume of amniotic fluid. The uterine horn was replaced in the abdomen and the peritoneum sutured with silk. The abdominal wall was closed with Michel clips and the mice allowed to recover in a warm environment. Either the left or the right uterine horn was used.

The females were killed on d 19 of gestation by cervical dislocation. The uterine horns were removed, then the fetuses were dissected free from their extraembryonic membranes and transferred into phosphate buffered saline. All fetuses were immediately examined under a dissecting microscope for evidence of morpho-

logical anomalies. Their crown–rump lengths were measured and the fetuses were then blotted free of surface saline and weighed. The fetuses were then killed by chloroform inhalation and immersion fixed at room temperature in Bouin's fluid for 24 h.

A representative selection of Bouin-fixed control and experimental fetuses were photographed to display the normal appearance of the limbs as observed in the control series, and the range of limb, tail and postural abnormalities observed in the experimental series.

Student's t tests and where numbers of observations allowed, z tests were used to analyse the data statistically.

RESULTS

Preliminary experiments to establish timing of amniotic sac puncture and optimal needle gauge which induces highest rates of palatal and limb abnormalities

To establish the most efficient method of producing palate and limb abnormalities, preliminary amniotic sac puncture experiments were carried out involving a small number of mice being subjected to this procedure on d 12, 13, 14, 15 or 16 of gestation, with either 21 or 25 gauge needles. The results of these preliminary experiments indicated that the earlier the amniotic sac puncture procedure was carried out, the higher the fetal mortality when assessed on d 19 of pregnancy. When amniotic sac puncture was carried out on d 12, only 6% of fetuses survived. Even in the control series, the survival rate was only about 50% (personal unpublished observations). The critical period for the production of palate and limb abnormalities was found to be on d 13 and 14. When this procedure was carried out on subsequent days of pregnancy, no palatal defects were seen, and the incidence of limb and tail abnormalities was low. When amniotic sac puncture with a 21 gauge needle was carried out on either d 13 or 14, frequent abnormalities of these 2 systems were encountered, without the induction of unacceptably high resorption rates. When a 25 gauge needle was used, however, the incidence of abnormalities induced, even when amniotic sac puncture was carried out on d 13 and 14 of pregnancy, was low. Therefore, the remaining experiments were carried out using a 21 gauge needle, with a total of 21 pregnant females being operated on at d 13 of gestation, and a further 17 on d 14 of gestation.

Findings from d 13 and 14 series, using 21 gauge needle

Survival rates, and overall incidence of palate, limb and tail abnormalities. Fetuses subjected to amniotic sac puncture on d 13 were more likely to die in utero, with a resorption rate of 47%, compared with a 19% resorption rate when this procedure was carried out on d 14; 97% of the d 13 and 90% of the d 14 controls survived. None of the control fetuses displayed any gross abnormalities. Of the experimental fetuses surviving, 70% of those from the d 13 series displayed at least one abnormality, whilst 48% of those treated on d 14 displayed at least one abnormality. The percentages of abnormalities encountered, as cited in the following text, refer to those fetuses that survived to d 19. The abnormalities observed were distributed in 3 principal categories:

Table 1. *Incidence of malformations induced by amniotic sac puncture carried out under general anaesthesia on either d 13 or 14 of pregnancy, using a 21 gauge needle. The analysis was performed on d 19 of pregnancy*

							Limb abnormalities				
							Forelimb		Hindlimb		
Day of pregnancy	Group	Total pregnant females	Total implants analysed	Total viable fetuses (%)	Total normal fetuses (%)	Palate defect	Right (%)	Left (%)	Right (%)	Left (%)	Tail abnormalities (%)
13	Experimental*	21	102	54 (53)	16 (30)	19 (35)	12 (22)	11 (20)	19 (35)	25 (46)	23 (43)
	Control†		86	83 (97)	83 (100)	—	—	—	—	—	—
14	Experimental*	17	83	67 (81)	36 (54)	18 (27)	7 (10)	5 (7)	15 (22)	17 (25)	13 (19)
	Control†		61	55 (90)	55 (100)	—	—	—	—	—	—

* Exposed to amniotic sac puncture; † embryos from contralateral uterine horns.

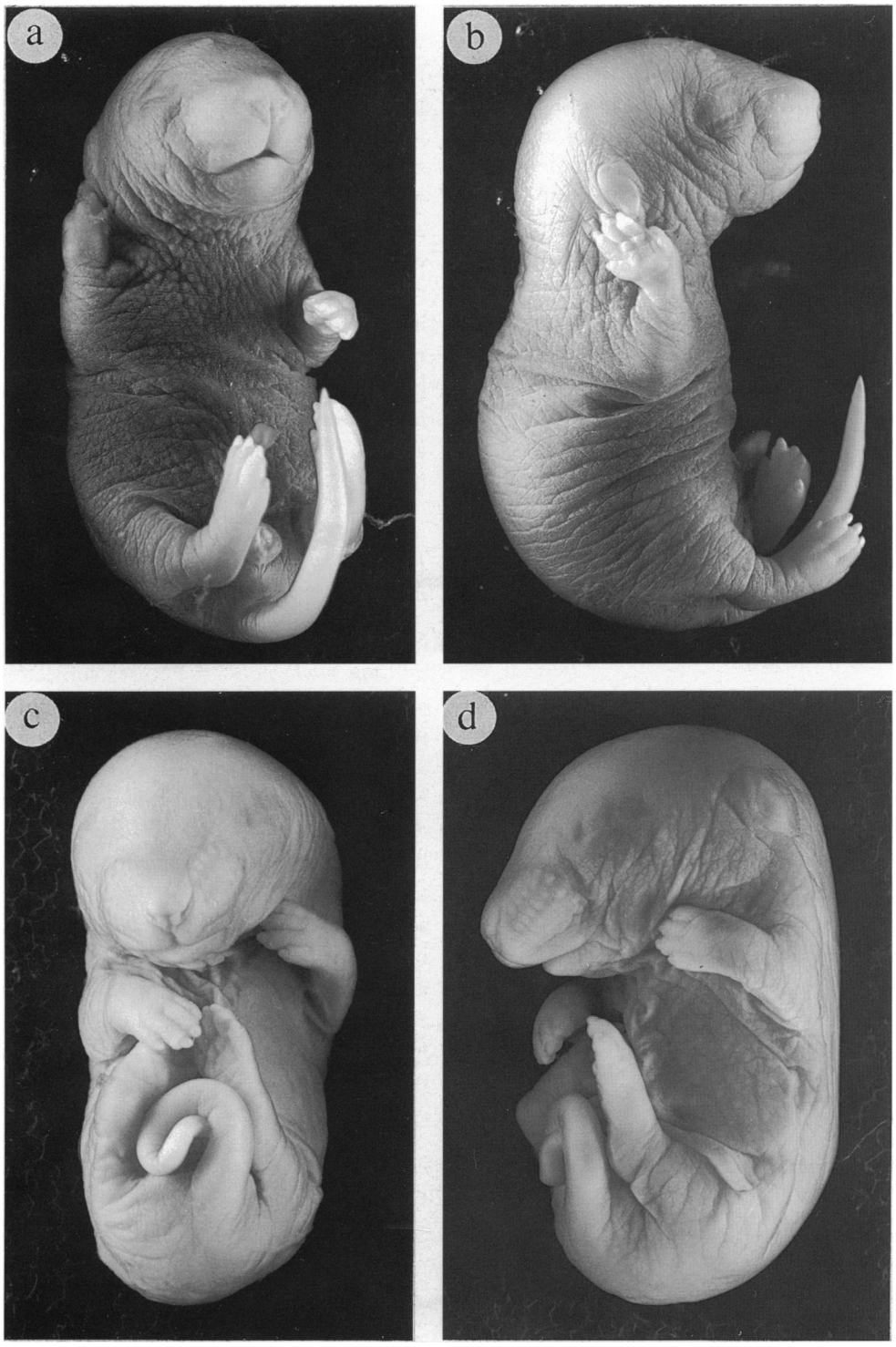

Fig. 1. Two experimental fetuses isolated on d 19 of gestation which display mild to moderate degrees of compression. Frontal (*a*) and lateral (*b*) views of a fetus which displays a mild degree of compression. Note the normal erect posture, but that the right fore limb is fused to the neck. The lower jaw profile is normal. Syndactyly of digits 3 and 4 of the right hind limb is present (for close-up view, see Fig. 3). Frontal (*c*) and lateral (*d*) views of a fetus which displays a moderate degree of compression. Note that all the limbs are abnormally positioned, that the tail is abnormally curved and syndactyly of digits 3 and 4 of the right fore limb is present. The lower jaw shows evidence of micrognathia. Approximate magnification ×4.

cleft palate, various types of limb abnormality, and anomalies of the tail. Details concerning these findings are given in Table 1.

External inspection of the experimental fetuses revealed varying degrees of compression. In the most severe examples, compression was typified by marked

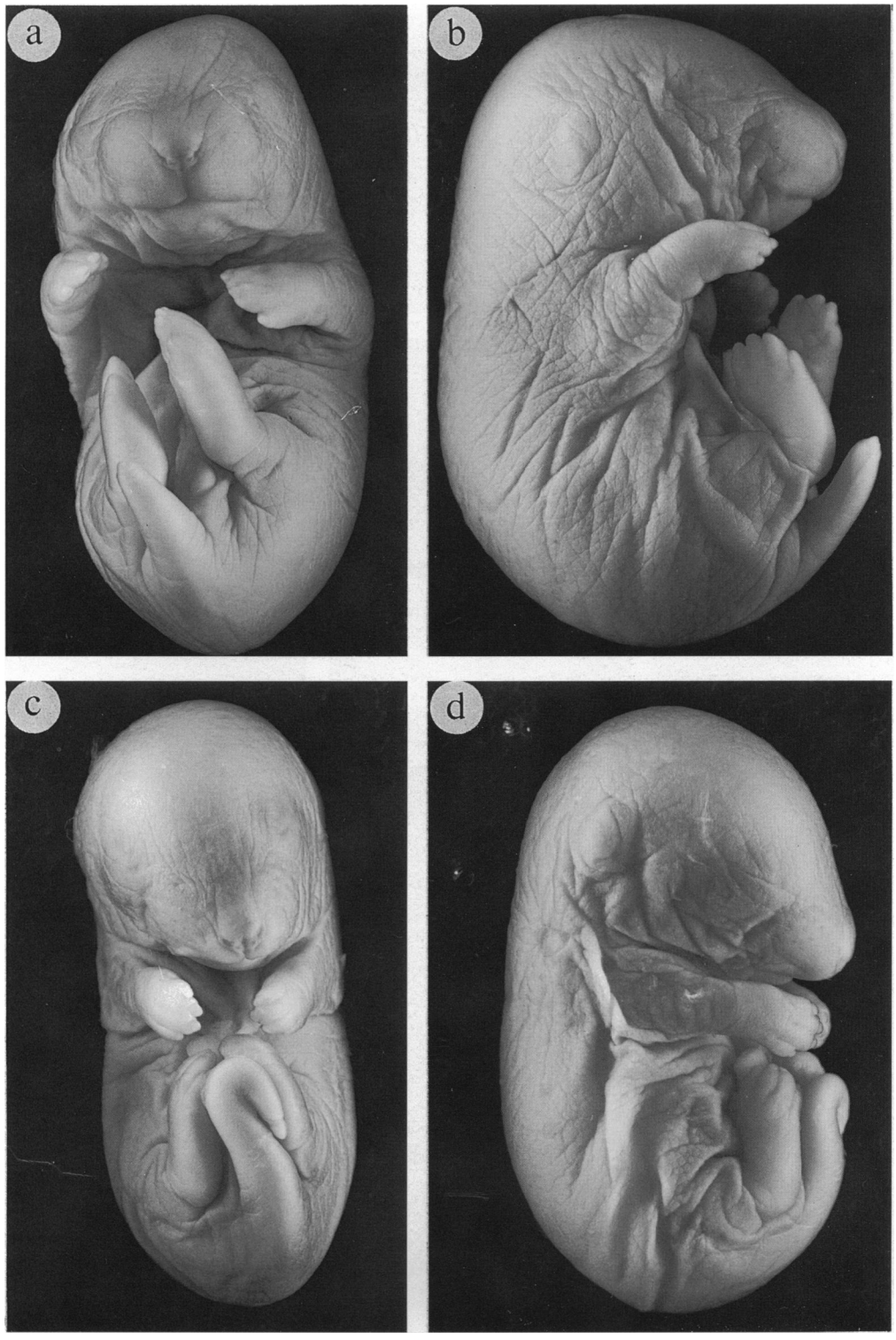

Fig. 2. Two experimental fetuses isolated on d 19 of gestation which respectively display moderate to severe and severe degrees of compression. Both have an abnormally deficient lower jaw profile suggesting contact under pressure with the thoracic wall. Both fetuses have an ovoid profile and tail deformities. Frontal (*a*) and lateral (*b*) views of a fetus with a moderate to severe degree of compression, which displays a transverse reduction deformity of the tail. The skin is markedly wrinkled, and the right forelimb displays syndactyly of digits 3 and 4. Frontal (*c*) and lateral (*d*) views of a fetus with a severe degree of compression. Note the abnormal curvature and transverse reduction deformity of the tail, as well as syndactyly involving digits 2 and 3 of the right fore limb. A severe degree of craniocaudal compression is also evident. Approximate magnification ×4.

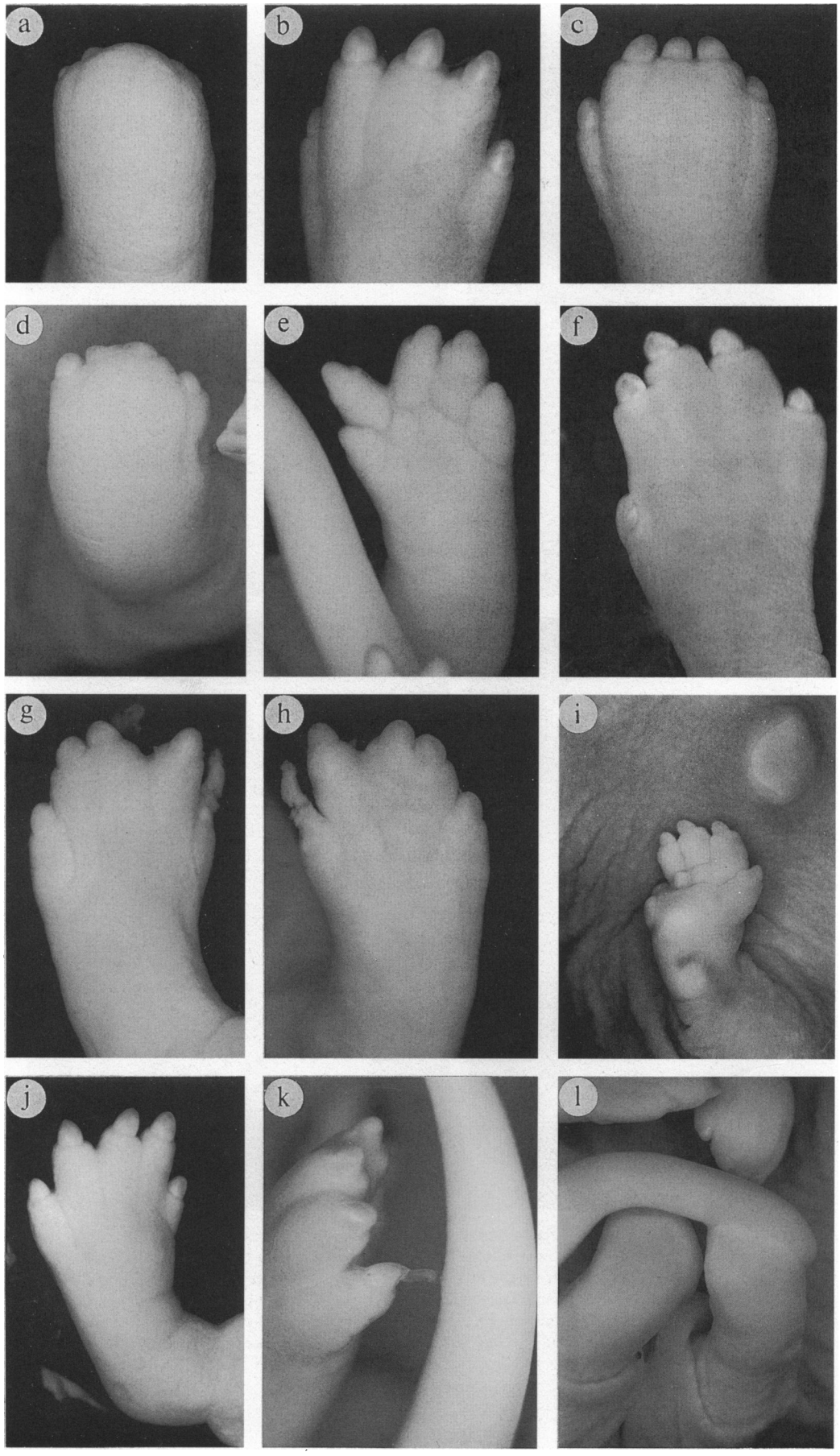

Fig. 3. For legend see opposite.

Table 2. *Varieties of abnormalities encountered* in 67 affected limbs of mouse fetuses subjected to amniotic sac puncture on the 13th day of gestation, and 44 affected limbs of mouse fetuses subjected to amniotic sac puncture on the 14th day of gestation*

Day of pregnancy		Limbs affected (%)	Adactyly	Syndactyly	Synonychia	Brachy-syndactyly	Diasto-dactyly	Ulnar/radial deviation	Talipes equinovarus	Fusion of limb to other parts
13	LFL	11 (16)	2	4	0	2	0	2	N/A	1 (tail)
	RFL	12 (18)	0	4	1	3	0	5	N/A	0
	LHL	25 (37)	1	10	2	7	3	N/A	6	1 (tail)
	RHL	19 (28)	0	7	3	10	1	N/A	3	0
	Total	67	3	25	6	22	4	7	9	2
	%		4	37	9	33	6	30	20	3
14	LFL	5 (11)	0	2	0	2	2	0	N/A	1 (neck)
	RFL	7 (16)	0	4	0	1	1	0	N/A	1 (neck)
	LHL	17 (39)	0	7	0	4	1	N/A	9	0
	RHL	15 (34)	0	3	0	2	0	N/A	11	0
	Total	44	0	16	0	9	4	0	20	2
	%		0	36	0	20	9	0	45	5

LFL, left fore limb; RFL, right fore limb; LHL, left hind limb; RHL, right hind limb; *multiple abnormalities in each limb have been counted separately.

wrinkling of the skin and 'moulding' of the head, body, limbs and tail, so that the fetus was ovoid in shape. In the other extreme, some fetuses from the experimental series were apparently normal. Examples of varying degrees of compression are illustrated in Figures 1 and 2. Cleft palate was present in 35% of fetuses operated on on d 13 and in 27% of those operated on on d 14. In these cases, opening the mouth revealed that the tongue was interposed between the palatal shelves. By dissecting the mandible and tongue free, a complete cleft of the posterior palate was observed. In addition, the tongue showed evidence of compression, consistent with the shape of the cleft. In all cases in which palatal defects were present, the primary palate was seen to be intact.

Limb abnormalities. Classification of the limb abnormalities proved more difficult than expected. Reference to the literature provided a multitude of descriptive terms with varying degrees of specificity (Hall et al. 1962; Henkel & Willert, 1969; Forfar & Arneil, 1985). Following the proposal by Froster & Baird (1992), it was decided to adopt a classification based on suggestions of the American Society of Surgeons of the Hand and of the International Society of Prosthetics and Orthotics (Swanson, 1976). One abnormality observed, however, did not correspond with any of the terms encountered. For this abnormality, the term *diastodactyly* was devised [from the Greek *diasta(nai)* to set apart + *daktyl(os)* finger], to cover those cases where there was lateral divergence of 2 adjoining digits. This contrasts with *ectrodactyly* where 2 digits may diverge because of the absence of all components of an intervening digit.

The overall incidence of limb abnormality was much greater in the d 13 compared with the d 14 experimental fetuses (61% compared with 39% ($P < 0.01$)). In both groups, hind limbs were highly significantly more frequently affected than the fore limbs (33% compared with 17% in d 13 fetuses ($P < 0.001$), and 37% compared with 13% in d 14 fetuses ($P < 0.01$)).

The limb abnormalities encountered were adactyly, syndactyly, synonychia, brachysyndactyly, diastodactyly, ulnar or radial deviation of the digits, talipes equinovarus and fusions of a limb to another part of the fetus. A representative example of each of these abnormalities is presented in Figure 3, and appropriate descriptions given in the figure legends. The relative frequencies of each of these abnormalities are detailed in Table 2. Syndactyly and brachysyndactyly

Fig. 3. Various types of limb deformities seen in experimental fetuses isolated on d 19 of gestation. (*a*) Adactyly. Lack of digits (dorsal aspect of hind limb). Note absence of recognisable digits, but evidence of small nail rudiments. (*b*) Syndactyly. Fusion of digits (dorsal aspect of hind limb). (*c*) Brachysyndactyly. Fusion and shortening of digits (dorsal aspect of hindlimb). (*d*) Synonychia (fusion of nails) and syndactyly (dorsal aspect of fore limb). (*e*) Diastodactyly. Lateral divergence of two adjacent digits (plantar aspect of hind limb). (*f*) Radial deviation (dorsal aspect of fore limb). (*g*) Diastodactyly and syndactyly (dorsal aspect of hind limb). (*h*) Plantar view of hind limb depicted in (*g*) showing presence of possible amniotic band (involving digit 1). (*i*) Fusion of limb to side of neck, just caudal to left ear (lateral view). (*j*) Talipes equinovarus. Medial deviation of paw region, so that the lateral border of the paw is directed inferiorly (lateral view). (*k*) Fusion of hind limb to tail by thin band (frontal view). (*l*) Fusion of left fore limb to tail (frontal view of specimen — rostral part of the fetus is directed to the right of the photograph). Approximate magnification: × 10.

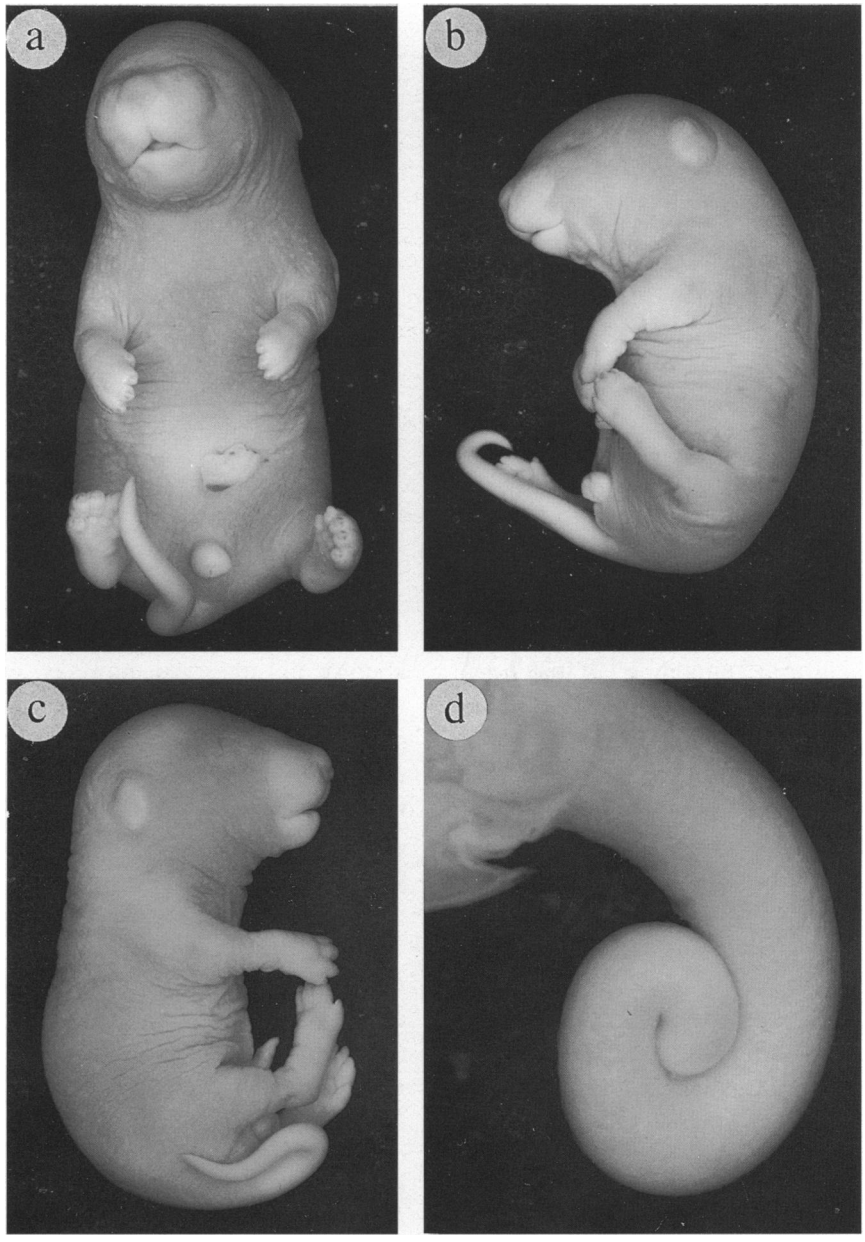

Fig. 4. Various types of tail abnormalities seen in experimental fetuses isolated on d 19 of gestation. (*a*) Frontal view of fetus with tail displaying 'kinked' appearance. (*b*) Lateral view of fetus with tail displaying distal 'hook'. Note also that the left hind limb displays an abnormal posture. (*c*) Lateral view of fetus with the tail showing a 'double-kink'. (*d*) Lateral view of tail showing an unusual spiral. Approximate magnification: *a–c*, ×4; *d*, ×10.

(mutually exclusive) and talipes equinovarus were the most frequent abnormalities produced, with incidences of 37, 33 and 20% in d 13 fetuses, respectively, and incidences of 36, 20 and 45% in d 14 fetuses, respectively.

Tail abnormalities. Fetal tails showed varying degrees of abnormal curvature and transverse reduction deformities. Tail abnormalities also proved more common in d 13 compared with d 14 fetuses (43 compared with 19% ($P < 0.01$)). Representative examples are depicted in Figure 4 (see also Figs 1, 2). Two other abnormalities occurred once each, namely exencephaly and omphalocele.

Detailed data analysis. When the 3 principal categories of abnormality were considered, it became apparent that most fetuses with cleft palate had an additional defect. Frequencies of combinations of the 3 principal categories of abnormality are presented in Table 3.

Data analysis of the fetuses revealed significant differences between the control and experimental crown–rump lengths and weights in both series

Table 3. *Combinations of various abnormalities observed in mouse fetuses subjected to amniotic sac puncture on either d 13 or 14 of pregnancy*

Day of pregnancy	Viable fetuses (%)	CP only	LA only	TA only	CP+TA	CP+LA	LA+TA	CP+LA+TA
13	54 (53)	3	8	2	0	4	9	12
14	67 (81)	3	8	1	1	7	4	7

CP, cleft palate; LA, limb abnormality; TA, tail abnormality.

Table 4. *Crown–rump lengths and weights of fetuses subjected to amniotic sac puncture on either d 13 or 14, that were viable at the time of analysis on d 19 of pregnancy*

Day of pregnancy		Normal* Exp.		Abnormal† Exp.		All Exp.		Control	
		Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
13	C–R	21.53	0.374	20.41	0.288	20.74	0.241	22.67	0.165
	Wt	1.072	0.019	1.069	0.021	1.070	0.016	1.133	0.011
	No.	16		38		54		83	
14	C–R	21.88	0.258	21.20	0.353	21.57	0.217	23.31	0.162
	Wt	1.150	0.013	1.084	0.031	1.120	0.016	1.203	0.013
	No.	36		30‡		66‡		55	

* No abnormal features present; † one or more abnormal features present; ‡ one exencephalic fetus excluded from the analysis. C–R, crown–rump length (mm); Wt, weight (g); S.E.M., standard error of the mean; Exp., experimental.

Table 5. *Significance values for crown–rump length data for d 13 and 14 series*

Day of pregnancy		Abnormal	Normal	All experimental	Control
13	Abnormal*	—	$P < 0.01$	—	$P < 0.01$
	Normal†	$P < 0.01$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—
14	Abnormal	—	$\underline{P > 0.05}$	—	$P < 0.01$
	Normal	$\underline{P > 0.05}$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—

The underlined P values indicate that no significant difference was found at the 5% level. * One or more abnormal features present; † no abnormal features present.

($P < 0.01$), with the experimental embryos being significantly lighter and smaller than controls. Additionally, differences were found between the crown–rump lengths of the d 13 fetuses that had one or more abnormality, and those experimental fetuses that appeared on gross inspection to be completely normal ($P < 0.01$). Similarly, the weights of the apparently normal and abnormal d 14 experimental fetuses differed significantly ($P < 0.05$) (see Table 4). A summary of the significant differences calculated is presented in Tables 5 and 6.

When the results of d 13 and 14 were pooled, 69 out of 121 (57%) fetuses showed at least one abnormality. Although the critical period in rats is d 15 (normal rat gestation ~ 22 d), this is equivalent, in developmental terms, to d 13/14 in the mouse (Witschi, 1962; Kaufman, 1992). The incidence of palatal, tail and most types of limb defect, with the exception of diastodactyly and talipes equinovarus (and brachysyndactyly which showed equal incidence), was higher on d 13 than d 14 (overall incidence 61%, compared with 39% ($P < 0.01$), respectively).

Table 6. *Significance values for fetal weight data for d 13 and 14 series*

Day of pregnancy		Abnormal	Normal	All experimental	Control
13	Abnormal*	—	$\underline{P > 0.05}$	—	$P < 0.01$
	Normal†	$\underline{P > 0.05}$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—
14	Abnormal	—	$P < 0.05$	—	$P < 0.01$
	Normal	$P < 0.05$	—	—	$P < 0.01$
	All experimental	—	—	—	$P < 0.01$
	Control	$P < 0.01$	$P < 0.01$	$P < 0.01$	—

The underlined P values indicate that no significant difference was found at the 5% level. *One or more abnormal features present; † no abnormal features present.

DISCUSSION

Analysis of the findings

Oligohydramnios, following amniotic sac puncture or amniocentesis, has been used as a teratogenic model in rats (for references, see Introduction). The resulting phenotypic disturbances may be termed the amniotic sac puncture syndrome. The key features are cleft palate and limb abnormalities. The present study has demonstrated that when amniotic sac puncture is carried out on either d 13 or d 14 of pregnancy in mice, cleft palate and limb abnormalities can also be produced reliably in this species.

In the present study, the frequency of palate, limb and tail abnormalities was greater when amniotic sac puncture was undertaken on d 13 compared with d 14 of pregnancy. It is possibly relevant to note that the limb defects associated with *cvs* appeared to show a similar degree of temporal specificity: 56–66 d in one study (Firth et al. 1991) and 63–79 d in another (Burton et al. 1993). The stage of development of mouse embryos at the time of amniotic sac puncture in the present study is equivalent to about 50–60 d postovulation, or Carnegie stages 20–23 of human embryonic/fetal development (O'Rahilly & Müller, 1987; Kaufman, 1992). These findings may indicate that a common causal mechanism is involved.

In this study, hind limbs were more frequently affected than forelimbs ($P < 0.01$). Several explanations for this phenomenon present themselves. If the limbs are affected by direct pressure, it is possible that the forelimbs are 'protected' to some degree because of their anatomical position, being located in close proximity to the overhanging fetal head (Kennedy & Persaud, 1977). It is, however, likely to be of greater importance that the fore limbs are about 12 h more advanced developmentally than the hind limbs at about d 12.5–13 in the mouse (Kaufman, 1992), and being less well developed structures, the hind limbs are likely to be more sensitive to teratogenic stimuli.

Due to the lack of consensus regarding the nomenclature of limb abnormalities, it is difficult to make a direct comparison between the relative frequencies of the specific abnormalities noted in this study with those reported by others. However, the findings of this study appear to be similar to those of the only comparable report in the literature that we have so far located (Singh et al. 1974) in which a relatively large number of experimental fetuses were observed, a proportion of which developed limb abnormalities following amniotic sac puncture.

Although tail defects have been observed very occasionally in rat fetuses following amniotic sac puncture, these have been described as being either 'scoliotic' (DeMyer & Baird, 1969) or 'kinked' (Love & Vickers, 1972). Houben (1984) described haemorrhage in rat tails shortly after amniocentesis, though it is of interest that he states that '... haemorrhage can be extensive and never impairs development.' In none of these earlier studies, however, was the incidence of tail defects observed quantified in any way. The present study has found a significant incidence of abnormal curvature of the tail following amniotic puncture. In addition, a number of tails displayed transverse reduction deformity (see Fig. 2) which has not, as far as we have been able to establish, been reported previously in the literature.

Data from Kaufman (1992, and unpublished) suggest that the control fetuses in this study were normal with respect to both crown–rump length and weight when analysed on d 19 of gestation. Singh et al. (1974) reported that the difference in weight between the control and experimental groups in their study was significant. The present study confirms the latter observation in the mouse model ($P < 0.01$), and additionally observed a highly significant reduction in

crown–rump length following amniotic sac puncture ($P < 0.01$), which has not been reported previously.

Hypotheses regarding the possible mechanisms involved in the production of palatal, limb and tail abnormalities

Palatal abnormalities. Poswillo (1966; Poswillo & Roy, 1965) noted micrognathia and glossoptosis in rat fetuses with cleft palate following amniotic sac puncture, and suggested an analogy with what at the time was termed the human Pierre Robin syndrome (or 'sequence;' see Robin, 1929, and Jones, 1988), in which the triad of cleft palate, micrognathia and glossoptosis were observed. Interestingly, it was this group that showed the greatest degree of concurrent limb abnormalities (Routledge, 1960; Smith & Stowe, 1961).

The association between craniofacial and limb abnormalities reported in this study has been a consistent finding in other studies of amniotic sac puncture in the rat (Poswillo, 1966; DeMyer & Baird, 1969; Love & Vickers, 1972; Singh & Singh, 1973; Singh et al. 1974; Kino, 1975; Kennedy & Persaud 1977; Houben, 1980, 1984; Houben & Huygen, 1987); however, there appears to be no literature regarding the relationship between amniotic sac puncture and limb abnormalities in the mouse. This study found a high incidence of fetuses with cleft palate, most of which also possessed limb abnormalities. Indeed, the latter was higher than might have been predicted from the clinical studies of infants formerly diagnosed as having the Pierre Robin sequence, where the incidence of limb defects was about 20% (Smith & Stowe, 1961).

The current concept of the common phenotype of the Pierre Robin sequence does not include limb malformations, so that the phenotype now exclusively relates to the craniofacial lesions indicated above. The aetiological heterogeneity of the syndrome is also now well established, and those infants previously ascribed to this syndrome would now be subdivided into a number of other disorders. About 30% of infants with the Robin sequence would now, for example, be diagnosed as having Stickler syndrome (Gorlin et al. 1990).

Limb and tail abnormalities. Little unanimity exists concerning the mechanism(s) underlying the formation of limb abnormalities following amniotic sac puncture (Love & Vickers, 1972; Kino, 1975; Kennedy & Persaud, 1977; Houben, 1984), and few experimental studies are available which shed light on this question, although Miller (1983) proposed that certain human limb abnormalities could result from intrauterine constraint, a similar mechanism to that proposed by Poswillo (1966, 1968) and others following amniotic sac puncture. In those studies in which the limbs were examined shortly after amniotic sac puncture, most researchers have reported the presence of localised haemorrhages. These, it was suggested, might have resulted in tissue destruction (Love & Vickers, 1972; Kino, 1975; Kennedy & Persaud, 1977). Clearly, while a vascular aetiology for the limb abnormalities observed seems a likely possibility, the exact mechanism(s) involved has yet to be established.

The tail defects observed, which range from complete amputations at different levels, to various degrees of lateral curvature, have not previously been reported. Their incidence in the present study (36% in the 13 d series, and 29% in the 14 d series), being commonly found in association with abnormalities of the limbs, suggests a common causal mechanism. It is interesting to note that a recently recognised mouse mutant that characteristically displays haemorrhage and subsequent transverse reduction of the tail, has been proposed as a model for a variety of human syndromes which display transverse reduction defects of the limb (Seller & Wallace, 1993).

General observations

Crane & Rholand (1986) drew attention to the potential risk of persistent amniotic fluid leakage after amniocentesis carried out during human pregnancy. Since the late 1980s, numerous reports have appeared (see Introduction) which clearly demonstrate a relationship between *cvs* and craniofacial and limb abnormalities, though the underlying mechanism(s) involved have yet to be established. It is our belief that in at least a proportion of these cases the abnormalities observed may have resulted from the inadvertent puncture of the amniotic sac during *cvs*, where there is either a substantial fluid loss at the time of *cvs*, or persistent but insidious subsequent loss of amniotic fluid.

Leakage of amniotic fluid is said to occur in at least 1–2% of all cases of amniocentesis carried out during the second trimester of pregnancy for the purpose of genetic analysis, but this is believed to resolve spontaneously within 48 h (Crane & Rholand, 1986). More persistent leakage, however, may result in spontaneous abortion or the premature onset of labour, and has in addition been associated with the birth of infants with limb deformities (Fleischer et al. 1991). No information is available as to whether the

membranes spontaneously repair, how long it takes for this to occur (if at all), and whether this depends on the stage of gestation when puncture of the amniotic sac occurs. Indeed, little is known at the present time of the factors that control the production/replenishment of amniotic fluid or of the importance or otherwise of maintaining the hydrostatic pressure which is normally present within this compartment.

The overt leakage of amniotic fluid following *cvs* has occasionally been reported. Thus Hogge et al. (1986) indicated that in their study of 1000 cases of *cvs*, the incidence was 0.2%, while in 5 other cases (i.e. 0.5%) ultrasonic evidence of severe oligohydramnios was noted with ...'almost a complete lack of amniotic fluid', when ultrasonography was carried out between 16 and 20 wk of gestation. These authors furthermore noted that in these 5 cases 'there was no clinical history to suggest overt rupture of the membranes or leakage of amniotic fluid'. The overall incidence of amniotic fluid leakage in the MRC trial was 0.43% (see MRC Working Party on the Evaluation of Chorionic Villus Sampling, 1991; Editorial, 1991). Amniotic fluid leakage may in fact occur considerably more often that noted by either the patient or her clinician.

While, clearly, it is only very rarely possible to establish unequivocally what the underlying cause(s) of a particular malformation may be, it seems reasonable to suggest that most of the reported cases of infants with oromandibular-limb hypogenesis malformations probably resulted either from vascular interruption, possibly due to detachment of a localised part of the placenta and formation of subchorionic haematoma or, as we would wish to propose, they may occasionally arise as a consequence of the postural or other problems that might result from the inadvertent puncturing of the amniotic sac and subsequent leakage of amniotic fluid from this compartment.

Clearly, there are differences between the experimental model described here and early *cvs* in humans. In the model, a substantial loss of amniotic fluid is seen to occur during amniotic sac puncture, whereas any leakage of amniotic fluid that does occur during *cvs* is only very occasionally recognised. The findings from our study do, we believe, indicate that this might be a useful model system to analyse the mechanism(s) involved in inducing the limb defects, in particular, seen in a high proportion of the mouse embryos exposed to amniotic sac puncture.

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